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Increased tau phosphorylation and receptor for advanced glycation endproducts (RAGE) in the brain of mice infected with *Leishmania amazonensis*

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ABSTRACT

Leishmaniasis is a parasitosis caused by several species of the genus *Leishmania*, an obligate intramacrophagic parasite. Although neurologic symptoms have been observed in human cases of leishmaniasis, the manifestation of neurodegenerative processes is poorly studied. The aim of the present work was to investigate if peripheral infection of BALB/c mice with *Leishmania amazonensis* affects tau phosphorylation and RAGE protein content in the brain, which represent biochemical markers of neurodegenerative processes observed in diseases with a pro-inflammatory component, including Alzheimer's disease and Down syndrome. Four months after a single right hind footpad subcutaneous injection of *L. amazonensis*, the brain cortex of BALB/c mice was isolated. Western blot analysis indicated an increase in tau phosphorylation (Ser^{396}) and RAGE immunocontent in infected animals. Brain tissue TNF-α, IL-1β, and IL-6 levels were not different from control animals; however, increased protein carbonylation, decreased IFN-γ levels and impairment in antioxidant defenses were detected. Systemic antioxidant treatment (NAC 20 mg/kg, i.p.) inhibited tau phosphorylation and recovered IFN-γ levels. These data, altogether, indicate an association between impaired redox state, tau phosphorylation and RAGE up-regulation in the brain cortex of animals infected with *L. amazonensis*. In this context, it is possible that neurologic symptoms associated to chronic leishmaniasis are associated to disruptions in the homeostasis of CNS proteins, such as tau and RAGE, as consequence of oxidative stress. This is the first demonstration of alterations in biochemical parameters of neurodegeneration in an experimental model of *Leishmania* infection.

1. Introduction

Leishmaniasis is a parasitosis caused by several species of the genus *Leishmania*, an obligate intramacrophagic parasite. Endemic leishmaniasis transmission occurs in at least 98 countries (Alvar et al., 2012). There are three main human syndromes caused by *Leishmania*: cutaneous disease, the least severe form of disease; mucocutaneous disease, which can be due the extension or metastasis of local skin lesions; and visceral leishmaniasis, also known as Kala-azar, the most severe form (Desjeux, 2004). The outcome of each is determined by the species of infecting parasite and the genetic susceptibility of the host (McGwire and Satoskar, 2013). *Leishmania amazonensis* causes different diseases depending on the host and parasitic virulence factors (Souza et al., 2011). Commonly, *L. amazonensis* infection is more associated to cutaneous leishmaniasis (Murray et al., 2005), but parasites have been also isolated from patients with the entire spectrum of the disease, including localized and diffuse cutaneous lesions, mucosal and visceral leishmaniasis (Barral et al., 1991). Recently, *L. amazonensis* was also associated with disseminate cutaneous leishmaniasis, an intermediate clinical form (David and Craft, 2009).

Although neurologic symptoms have been largely observed in human cases of the disease, the manifestation of degenerative

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processes associated with the central nervous system (CNS) in leishmaniasis is poorly studied. Cutaneous leishmaniasis is known to cause a peripheral neuropathy by direct or close parasite involvement with the nerve or nerve sheath (Petersen and Greenlee, 2011). In this form of the disease, parasites in the skin and draining lymph nodes are thought to cause an endocrine imbalance as a consequence of cytokines action on the CNS (de Moura et al., 2005). More recently, patients with cutaneous leishmaniasis were reported to exhibit an immune–endocrine imbalance with reduction of plasma levels of dehydroepiandrosterone-S, prolactin and testosterone (Baccan et al., 2011). Together with the observation that *L. amazonensis* may cross the blood–brain barrier and induce significant pathologic changes in the CNS (Abreu-Silva et al., 2003), these data indicate that relevant neurodegenerative processes may occur in the course of leishmaniasis.

The microtubule-stabilizing protein tau is mainly expressed in central nervous system (CNS) neurons and is essential for axon architecture and synaptic function. Initially, the aberrant hyperphosphorylation of tau was observed to be associated with the formation of neurotoxic histological structures in the CNS known as neurofibrillary tangles, characteristic of Alzheimer's disease (AD) (Sonnen et al., 2008). Later, tau hyperphosphorylation was detected in at least twenty-two different CNS related pathologies, including prionic diseases, amyotrophic lateral sclerosis and Down's syndrome (Spires-Jones et al., 2009). These conditions have been referred to as "tauopathies", in which tau aberrant phosphorylation is thought to exert a causative role in synapse impairment and progression of neuronal cell death. Although the molecular detailing of the steps coupling tau aberrant phosphorylation and neuronal death are currently not completely understood, it is clear that disruption of tau homeostasis is associated with an impairment of neural circuits and cognitive deficits (Gendron and Petrucelli, 2009).

The receptor for advanced glycation endproducts (RAGE) is a multiligand membrane receptor that exerts crucial roles in the development of chronic inflammatory processes (Srikanth et al., 2011). RAGE was initially observed to be associated with late diabetic complications, where the accumulation of advanced glycation endproducts (AGE) on the endothelial surface triggers its expression and activation. Further studies have characterized RAGE as a damage-associated molecular pattern (DAMP) receptor, as other molecules with pro-inflammatory and pro-apoptotic activities were observed to act as RAGE ligands (Coughlan et al., 2007). These include the extracellular forms of HMGB1 and members of the S100/calgranulin family, such as S100B and S100A7 (Bopp et al., 2008; Leclerc et al., 2007). RAGE activation induces the expression of pro-inflammatory cytokines and NADPH oxidase activation, which in turn stimulate reactive species (RS) production, causing oxidative damage to biomolecules and sustaining local inflammation and tissue damage (Maczurek et al., 2008). In the brain, a prominent role of RAGE in the contribution to neurodegenerative processes has been emerging since the earlier observations that the β-amyloid peptide function as a RAGE ligand (Arancio et al., 2004; Du Yan et al., 1997). Since then, extensive data have been indicating a key role for RAGE in the chronic pro-inflammatory axis responsible for the progression of neuronal death observed in AD (Yan et al., 2009). On the other hand, the involvement of RAGE in other neuroinflammatory states potentially associated with neurodegeneration is poorly studied. In the present work, we investigated the phosphorylation of tau and the modulation of the immunocontent of RAGE in the brain cortex of BALB/c mice infected with *L. amazonensis*. We also analyzed oxidative stress and inflammatory parameters in order to study the possible relationship of RS production and inflammation in the modulation of neurodegeneration parameters in leishmaniasis.

2. Materials and methods

2.1. Chemicals

Glycine, H_2O_2 (hydrogen peroxide), catalase (CAT, EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1), thiobarbituric acid, epinephrine, AAPH (2,2′-azobis[2-methylpropionamidine]dihydrochloride), trichloroacetic acid (TCA), 2,4-dinitrophenylhydrazine (DNPH), 5,5′-dithionitrobis 2-nitrobenzoic acid (DTNB), Bile salts, sodium dodecyl sulfate, DNP polyclonal antibody and monoclonal TNF-α antibody were purchased from Sigma–Aldrich®(St. Louis, USA). Electrophoresis and immunoblot reagents were from Bio-Rad (Hercules, USA), GE Healthcare Brazilian Headquarter (São Paulo, Brazil) and Sigma–Aldrich®RAGE polyclonal antibody, phosphorylated tau polyclonal antibody, total tau polyclonal antibody, β-actin polyclonal antibody, IL-1β polyclonal antibody and anti-rabbit immunoglobulin linked to peroxidase were from Cell Signalling technology®(Beverly, USA). ELISA microplates were from Greiner Bio-One (Monroe, USA) and ELISA TMB spectrophotometric detection kit was from BD Biosciences (San Diego, USA). Immunoblot chemiluminescence detection was carried out with the West Pico detection kit from Thermo Scientific Pierce Protein Biology Products (Rockford, USA). MilliQ-purified H_2O was used for preparing solutions. All other reagents used in this study were of analytical or HPLC grade.

2.2. Ethics statement

All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 80-23 revised 1996) and the Brazilian Society for Neuroscience and Behavior recommendations for animal care. The experimental protocols were approved by the Oswaldo Cruz Foundation *Committee* of Ethics for the Use of Animals (CEUA–Fiocruz) protocol number P-36/11-3.

2.3. Parasite strain and infection

L. amazonensis (MHOM/BR/75/JOSEFA) were maintained by regular passage in BALB/c mice. Amastigotes were purified from the footpad lesions of mice as previously described (Barbieri et al., 1993). Female BALB/c mice (6 weeks old) were obtained from Centro de Criação de Animais de Laboratório (CECAL)–Fiocruz, Rio de Janeiro, RJ, Brazil, and injected subcutaneously in the right hind footpad with 10^5 amastigotes (Arrais-Silva et al., 2006). Four months post-infection the animals were euthanized, the brain cortex was removed and maintained in liquid nitrogen until the assays were performed. Additional groups of mice received a systemic antioxidant therapy (*N*-acetylcysteine 20 mg/kg b.w., one daily injection, i.p.) for five consecutive days before euthanasia. During the post-infection period, animals show apathy and diminished eating behavior. To estimate parasite burden in the lesions, the entire infected footpads were removed and amastigotes were recovered from the lesions and counted. Inflammatory parameters in liver and serum (TNF-α and IL-1β) were measured to confirm systemic inflammation. In all assays five animals per group ($n = 5$) were utilized.

2.4. Antioxidant enzymes activities

Samples were homogenized in phosphate buffer (PB) 50 mM (KH_2PO_4 and K_2HPO_4, pH-7.4) and the protein content was determined by Bradford method (Bradford, 1976). Catalase (CAT; E.C. 1.11.1.6) activity was evaluated by following the rate of decrease in hydrogen peroxide (H_2O_2) absorbance in a spectrophotometer

at 240 nm (Aebi, 1984). Results are expressed as units of CAT/mg of protein. The activity of superoxide dismutase (SOD; EC 1.15.1.1) was measured by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a sample buffer; adrenochrome formation was monitored at 480 nm for 10 min (32 °C) in a spectrophotometer (Misra and Fridovich, 1972). Results are expressed as units of SOD/mg of protein.

2.5. Oxidative damage to proteins (carbonyl)

As an index of protein oxidative damage, the carbonyl groups were determined as previously described (Levine et al., 1990). The homogenate were divided into two aliquots of 300 μL (1 mg of protein). Proteins were precipitated by the addition of 150 μL of 20% TCA for 5 min on ice and centrifuged at 4000g for 5 min. The pellet was dissolved with 100 μL of sodium hydroxide (NaOH) (200 mM) and 100 μL of hydrochloric acid (HCl) (2 M) was added in blanks. DNPH (10 mM) was added for carbonyl groups derivatization. Samples were maintained for 30 min at room temperature. Proteins were precipitated with 20% TCA and washed three times with 500 μL of 1:1 ethanol:ethyl acetate with 15 min standing periods to remove the excess DNPH. Samples were dissolved in 200 μL of urea (8 M) pH 2.3, and the absorbance was read at 370 nm.

2.6. Western blot detection of dinitrophenyl (DNP)-labeled protein carbonyls

Tissue samples were homogenized in 1 volume of radio-immunoprecipitation assay (RIPA) buffer (20 mM Tris–HCl at pH 7.5, 150 mM NaCl, 1 mM Na_2 EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin), centrifuged (14,000g for 10 min at 4 °C) and the pellet proteins were quantified. Proteins were dissolved in 6% sodium dodecyl sulfate (SDS) and derivatized with an equal volume of DNPH 10 mM in 10% trifluoroacetic acid for 1 h (Shacter et al., 1994). Samples were subjected to SDS–polyacrylamide gel electrophoresis for Western blot detection of DNP-derivatized proteins with an antibody against DNP as described below.

2.7. Sulfhydryl groups quantification

Oxidative status of thiol groups were assessed by quantification of total reduced sulfhydryl (SH) groups in samples (Ellman, 1959). Briefly, for total SH content measurement, 60 μg sample aliquot was diluted in phosphate-buffered saline (PBS) (NaCl, Na_2HPO_4, KH_2PO_4), and 5,5′-dithionitrobis 2-nitrobenzoic acid (10 mM), and read in a spectrophotometer at 412 nm after 60 min of incubation in room temperature.

2.8. Index of lipid peroxidation (TBARS)

The quantification of thiobarbituric acid reactive substances (TBARS) was performed for evaluation of an index of lipoperoxidation, as previously described (Draper and Hadley, 1990). Brain cortex tissue was homogenized in ice-cold Tris–HCl 15 mM (pH 7.4) and reacted with an equal volume of 40% trichloroacetic acid (TCA), followed by centrifugation and addition of 0.67% TBA. Samples were then incubated at 100 °C for 25 min. After cooling, samples were centrifuged (750g/10 min) and supernatant absorbance was read at 535 nm.

2.9. Total reactive antioxidant potential (TRAP assay)

The total reactive antioxidant potential (TRAP) was used as an index of non-enzymatic antioxidant capacity. This assay is based on the quenching of peroxyl radicals generated by AAPH (2,2 azobis[2-amidinopropane]) by antioxidants present in a given sample (Lissi et al., 1992). Briefly, a chemical system that generates peroxyl radicals at a constant rate (an AAPH-containing buffer) is coupled to a luminescent reactant (luminol) which emits photons proportionally to its oxidation. The samples were homogenized with glycine buffer (pH-8.6). The reaction was initiated by injecting luminol to the 0.1 M glycine buffer containing AAPH that resulted in steady luminescence emission. Equal amounts of samples are then added to this reaction system, and the luminescence emission at the moment following this addition ($t = 0$) is recorded. This initial emission reflects the production of free radicals by AAPH at the first moment right after sample addition and is related to the endogenous oxidant state of the sample. Following incubation, the thermal decomposition of AAPH produces luminescence at a constant rate ("system"), and the presence of free radical scavengers in the added sample will decrease this rate according to its content of non-enzymatic antioxidants. We followed TRAP luminescence emission for 80 min and calculated the area under the curve (AUC) relative to the system without samples (which was considered as 100% of luminescence emission at all time points. The addition of the homogenate samples decreases or facilitates the luminescence emission proportionally to its redox state. The luminescence emission was recorded in a MicroBeta® luminescence counter (Perkin Elmer, USA).

2.10. TNF-α, IL-1β, IFN-γ, IL-6 and nitrotyrosine levels (ELISA)

TNF-α, IL-1β, IFN-γ, IL-6 and nitrotyrosine were quantified by indirect ELISA. Brain cortex homogenate was placed in ELISA plates. After 24 h incubation, plates were washed three times with Tween–Tris buffered saline (TTBS, 100 mM Tris–HCl, pH 7.5, containing 0.9% NaCl, and 0.1% Tween-20). Subsequently, 200 μL of anti-TNF-α, anti-IL-1β, anti-IFN-γ, anti-IL-6 or anti-nitrotyrosine (1:1000) were added and incubation was carried for 24 h at 4 °C. The plates were washed three times with TTBS and incubated with rabbit or mouse IgG peroxidase-linked secondary antibody (1:1000) for 2 h. After washing the plate three times with TTBS, 200 μL of substrate solution (TMB spectrophotometric ELISA detection kit) were added to each well and incubated for 15 min. The reaction was terminated with 50 μL/well of 12 M sulfuric acid stopping reagent and the plate read at 450 nm.

2.11. Immunoblot detection of phosphorylated tau and RAGE

To perform immunoblot experiments, the tissue was homogenated with 1X RIPA buffer, centrifuged (10,000g for 5 min at 4 °C) and the pellet proteins were measured by the Bradford method (Bradford, 1976). Laemmli-sample buffer (62.5 mM Tris–HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol) was added to complete volume according the protein content of each sample and equal amounts of cell protein (30 μg/well) were fractionated by SDS–PAGE and electro-blotted onto nitrocellulose membranes with Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell, Bio-Rad (Hercules, CA, USA) . Protein loading and electro-blotting efficiency were verified through Ponceau S staining, and the membrane was washed with Tween–Tris buffered saline (Tris 100 mM, pH 7.5, 0.9% NaCl and 0.1% Tween-20). Membranes were incubated 20 min at room temperature in SNAP i.d.® 2.0 Protein Detection System Merck Millipore (Billerica, MA, USA) with each primary antibody (anti-RAGE, anti-phospho-tau, anti-tau, anti-β-actin – 1:500 dilution range each) and then washed with TTBS. Anti-rabbit

or mouse IgG peroxidase-linked secondary antibody (1:5000 dilution range) was incubated with membranes for additional 20 min in SNAP i.d. system (Millipore, Billerica, MA, USA), washed again and the immunoreactivity was detected by enhanced chemiluminescence using Supersignal West Pico Chemiluminescent kit from Thermo Scientific (Luminol/Enhancer and Stable Peroxide Buffer). Densitometric analysis of the films was performed with Image J. software. Blots were developed to be linear in the range used for densitometry.

2.12. Statistical analysis

Statistical analysis was performed with GraphPad 5.0 software. Student's *t* test (two-tailed) was applied for simple comparisons between control and infected animals in each assay. For comparison of multiple means, ANOVA with Tukey's post hoc was performed. The results of measurements were expressed as mean ± standard error of the mean (SEM). Differences were considered significant when $p < 0.05$. The tissue protein content was measured by Bradford method for data normalization in all assays (Bradford, 1976).

3. Results

Tau may be phosphorylated by diverse protein kinases at approximately 25 different sites (Wang et al., 2013). However, phosphorylation of tau on Ser396 is one of the earliest events leading to neurofibrillary tangles formation in AD and Down syndrome and it was suggested to play a key role in the formation of paired helical filaments, the major component of neurofibrillary tangles (Mondragon-Rodriguez et al., 2014). We evaluated the content of phosphorylated and total tau isoforms in the brain cortex of mice infected by *L. amazonensis* by Western blot. An increase in the content of phospho-tau (Ser396) was detected, but the content of total tau was not changed (Fig. 1A). This result indicates that tau phosphorylation in infected animals is increased by a mechanism that is not associated with modulation of tau expression.

We next evaluated the immunocontent of RAGE in these samples of brain cortex. In animals infected by *L. amazonensis*, the total content of RAGE was significantly increased compared to control animals (Fig. 1B). In AD, both tau aberrant phosphorylation and RAGE up-regulation are strongly implicated in the molecular mechanisms underlying the progression of neuronal death (Li et al., 2012; Yan et al., 2009). RAGE has been suggested to be the key component of the chronic pro-inflammatory axis responsible for microglia activation and reactive species production that ultimately leads to neuronal cell death in AD and also in other neurodegenerative processes (Maczurek et al., 2008). For this reason, we next sought to evaluate parameters of inflammation and oxidative/nitrosative stress in the brain cortex of mice infected with *L. amazonensis*.

The levels of TNF-α and IL-1β were evaluated by ELISA. These cytokines are considered markers of acute pro-inflammatory activation and have been associated with modulation of tau phosphorylation and RAGE expression in some neuropathological states (Krstic et al., 2012; Roe et al., 2011). Although an increase in TNF-α levels was detected in other organs, such as liver (data not shown), no significant changes in the levels of both TNF-α and IL-1β were detected in brain cortex of infected animals (Fig. 2A and B). Thus, our results indicate that tau phosphorylation and RAGE upregulation are not associated with TNF-α and IL-1β in leishmaniasis.

We next evaluated parameters of oxidative and nitrosative damage in biomolecules of brain cortex samples from mice infected with *L. amazonensis*. We quantified levels of nitrotyrosine, a marker of peroxynitrite-mediated protein nitration, in brain cortex samples by ELISA. Besides, the quantification of free (reduced) thiol groups indicates the redox state of proteins and peptides containing sulfhydryl groups that may undergo oxidation or reduction and form disulfide bonds. No changes in the levels of nitrotyrosine and free thiol groups were observed between control and infected animals (Fig. 3A and B). On the other hand, the levels of TBARS were significantly increased in infected animals, suggesting an elevated status of brain lipid peroxidation (Fig. 3C). Besides, the Western blot analysis of DNP-reactive proteins indicated that protein carbonylation in infected animals was increased (Fig. 3D), which was also confirmed by quantification of carbonyl groups in the samples (Fig. 3E).

The antioxidant status of brain cortex samples was also analyzed. The activities of the antioxidant enzymes CAT and SOD were measured. A decrease in CAT activity was observed in infected animals, indicating impaired H_2O_2 detoxification capacity in brain cortex (Fig. 4A). SOD activity did not differ between control and infected animals (Fig. 4B). Also, the analysis of the non-enzymatic antioxidant status by the TRAP assay showed that mice infected by *L. amazonensis* had a decreased status in non-enzymatic antioxidant defense, indicating a diminished antioxidant capacity compared to control animals (Fig. 4C and D).

Finally, to verify whether tau phosphorylation was associated to the elevated status of oxidative stress in the brain cortex, mice infected with *L. amazonensis* were subjected to a daily i.p.

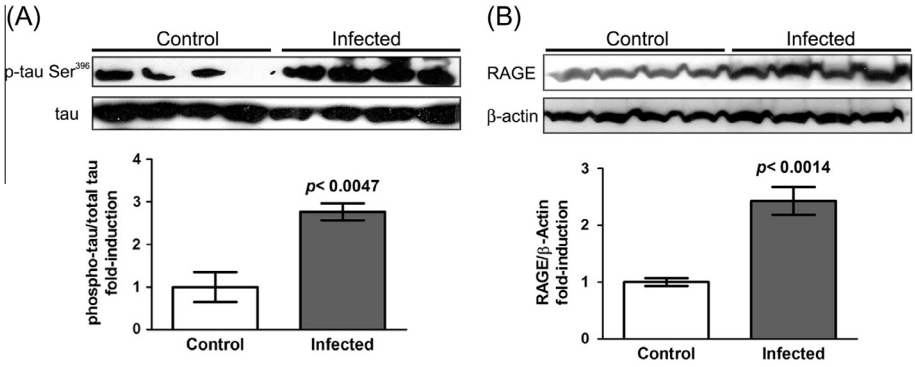

Fig. 1. Tau phosphorylation and RAGE content in brain cortex of mice infected with *L. amazonensis*. Four months after infection, brain cortex tissue from infected and control animals was removed and proteins were subjected to SDS–PAGE/Western blot analysis. (A) Representative immunoblots of tau phosphorylated at Ser396 (p-tau Ser^{396}, upper panel) and total tau (lower panel) from four control and four infected animals. Bar graph corresponds to mean ± SEM quantification values of the p-tau/total tau ratio from all samples. (B) Representative immunoblots of RAGE content from four control and four infected animals (upper panel). Lower panel corresponds to β-actin immunocontent used as control for protein constitutive expression. Bar graph corresponds to mean ± SEM quantification values of the RAGE/β-actin ratio from all samples. Values for *p* depicted were obtained by applying two-tailed student's *t* test.

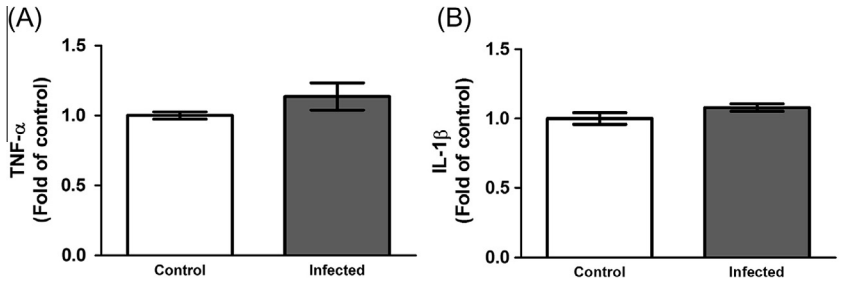

Fig. 2. Levels of TNF-α and IL-1β in brain cortex from mice infected with *L. amazonensis*. Four months after infection, brain cortex tissue from infected and control animals was removed and proteins were subjected to ELISA detection of (A) TNF-α and (B) IL-1β internal levels. Values represent mean ± SEM of five samples from each group. No statistical differences were detected using two-tailed student's *t* test (for $p = 0.005$).

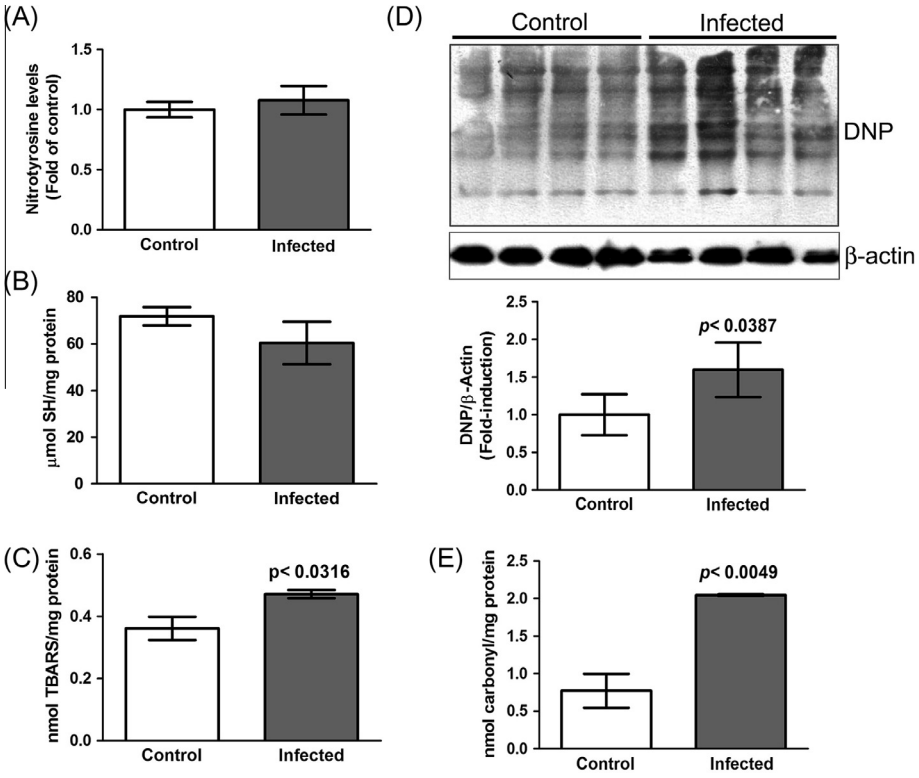

Fig. 3. Parameters of protein oxidative and nitrosative modifications in brain cortex from mice infected with *L. amazonensis*. (A) Protein samples from control and infected animals were subjected to nitrotyrosine detection by ELISA. (B) Free thiol (sulfhydryl; SH) groups quantification in brain cortex samples from control and infected animals. (C) Quantification of thiobarbituric acid reactive substances was performed as an index of lipoperoxidation. (D) Western blot detection of carbonyl groups was also performed by analysis of total protein fraction of samples previously derivatized with DNP. Polyclonal anti-DNP was used to detect DNP-carbonyl derivatized proteins (upper gel panel) and β-actin immunocontent used as control for protein constitutive expression (lower gel panel). Bar graph corresponds to mean ± SEM quantification values of the DNP/β-actin ratio from all samples. (E) Spectrophotometric quantification of carbonyl groups was performed in the same samples. Bar graphs in A, B, C and E correspond to mean ± SEM quantification values from all samples. Values for *p* were obtained by applying two-tailed student's *t* test and are shown only when $p < 0.005$.

administration with the antioxidant NAC (20 mg/kg) for five consecutive days before euthanasia. Infected animals that received NAC had decreased levels of phosphorylated tau compared to infected animals that did not receive antioxidant therapy (Fig. 5A and B), indicating a role for reactive species in tau phosphorylation. Additionally, we compared the levels of IFN-γ and IL-6, plus TNF-α and IL-1β, between control and infected animals receiving NAC. The levels of IFN-γ were decreased in infected animals, while the infected animals that received NAC recovered IFN-γ levels to control values (Fig. 5C), which indicates a role for oxidative stress in the modulation of IFN-γ in the brain cortex of animals infected with *L. amazonensis*. We did not observe any effect on IL-6 levels at any group of animals (Fig. 5D). Besides, infected animals receiving NAC presented decreased TNF-α levels compared to infected animals that did not receive antioxidant therapy (Fig. 5E). No variations in IL-1β levels were observed in all groups (Fig. 5F).

4. Discussion

Oxidative stress is an important factor in the course of *Leishmania* infection. *L. amazonensis* was reported to contain a cluster of genes expressing META1 and META2 proteins, which were found to be responsible for resistance of the parasite against heat shock and oxidative stress (Ramos et al., 2011). These characteristics probably evolved as a parasite's strategy of resistance against the oxidizing agents generated by inflammatory cells aimed to kill intracellular and extracellular pathogens. However, depending on

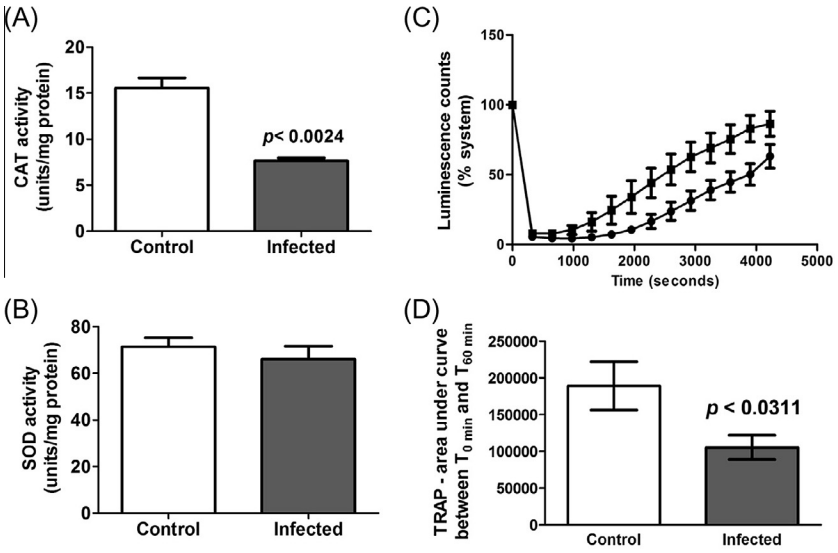

Fig. 4. Antioxidant capacity in brain cortex samples from mice infected with *L. amazonensis*. The activities of antioxidant enzymes and the non-enzymatic antioxidant potential in brain cortex samples from animals 4 months after infection were measured. (A) Catalase (CAT) activity. (B) Superoxide dismutase (SOD) activity. (C) Total reactive antioxidant parameter (TRAP) kinetic assay; square dots represent control and circle dots represent samples from infected animals. (D) Quantification of the area under the curve between time points 0 min and 60 min of the kinetic assay (arbitrary units are shown). Bar graphs correspond to mean ± SEM quantification values from all samples. Values for p were obtained by applying two-tailed student's t test and are shown only when $p < 0.005$.

the parasite's ability to cope with the oxidative stress generated by the host, the inflammatory response may be further intensified in order to kill more resistant micro-organisms. The intensification of the production of oxidizing agents during this response may affect cells and tissues of the host, thus contributing to further development of the disease. The observation of oxidative damage to proteins in the CNS of mice infected with *L. amazonensis* reported here may bring new information for the understanding of neurological symptoms in cutaneous leishmaniasis and related parasitic infections. In previous studies, alterations in the redox state of liver were observed only in visceral leishmaniasis caused by infection of hamsters with *Leishmania chagasi* (Oliveira and Cecchini, 2000).

Oxidative stress in patients and experimental models of leishmaniasis is generally analyzed in the context of infected cells or focusing on the microenvironment (tissue) of the injury. Granulomatous inflammatory reaction is associated with the presence of amastigotes within macrophages, reflecting in the function of organs such as liver, spleen, lymph nodes and bone marrow. Cells of the mononuclear phagocyte system are present in these organs (Baneth and Aroch, 2008; Engwerda et al., 2004). In a previous study conducted with the same animal model and infection protocol used here, it was observed that BALB/c mice infected with *L. amazonensis* displayed inflammatory infiltrates of mononuclear cells and neutrophils without parasites in the meninges and the presence of macrophages containing parasites in the cerebral parenchyma (Abreu-Silva et al., 2003). However, as observed in studies with human subjects, the mere presence of parasites in skin and draining lymph nodes (as in localized cutaneous leishmaniosis) affects several neuroendocrine axes, and these effects are believed to emerge as consequences of the actions of pro-inflammatory factors released by peripheral tissues on the CNS (Baccan et al., 2011).

Inflammation and oxidative stress are important components in several neurodegenerative conditions. In AD and related tauopathies, tau phosphorylation and RAGE up-regulation are unequivocally stimulated. These characteristics are strongly believed to be associated with the activation of RS production caused by factors that sustain a chronic state of local inflammatory activation in CNS. However, we did not observe any changes in the levels of TNF-α, IL-1β and IL-6 in the brain cortex of infected animals. These cytokines are associated with acute inflammatory responses, while RAGE is considered a marker of chronic pro-inflammatory development (Ibrahim et al., 2013). TNF-α promotes the induction of IL-6 and IL-1β, and high levels of these cytokines in macrophages of rats acutely infected with *Leishmania braziliensis* have been previously reported (Brelaz-de-Castro et al., 2012). However, in leishmaniasis, the action of these cytokines is more pronounced at either acute phases or advanced stages of the chronic disease (Oliveira et al., 2014). On the other hand, the levels of IFN-γ were decreased in the brain of infected animals, and the antioxidant therapy with NAC recovered IFN-γ status to control levels. IFN-γ is associated to the Th1 response, which is a necessary step of an adequate immune response to control the parasite. A Th1 predominant response is considered a good prognosis for control of the infection with most species of *Leishmania*, while Th2-predominant response is associated to the evolution of the disease (Pereira and Alves, 2008). Nonetheless, infections caused by *L. amazonensis* were observed to downregulate IFN-γ levels in lymph node cells to a greater extent compared to other *Leishmania* species (Maioli et al., 2004), and this was suggested to be part of a specific mechanism by which the parasite modulates the host immune system, as INF-γ-mediated induction of macrophage activation is essential for control of the parasite (Alexander and Bryson, 2005). Inhibition of IFN-γ production in leishmaniasis is directly associated to increased lesion size. The shift between Th1 and Th2 responses is influenced in different ways by the parasite at earlier and later stages of the disease, in order to couple the host immune response with the progression of the parasite's cycle and its change from amastigote to promastigote forms (Pereira and Alves, 2008).

Interestingly, infected mice that received NAC presented decreased TNF-α levels in the brain cortex compared to infected animals that were not treated with NAC. Variations in the levels of this cytokine were not observed between control and infected animals, but the antioxidant treatment was able to reduce this pro-inflammatory mediator in the brain of both control and infected mice. At systemic level, an increase in IL-4 production caused *L. amazonensis* promotes a Th2 response and downregulates

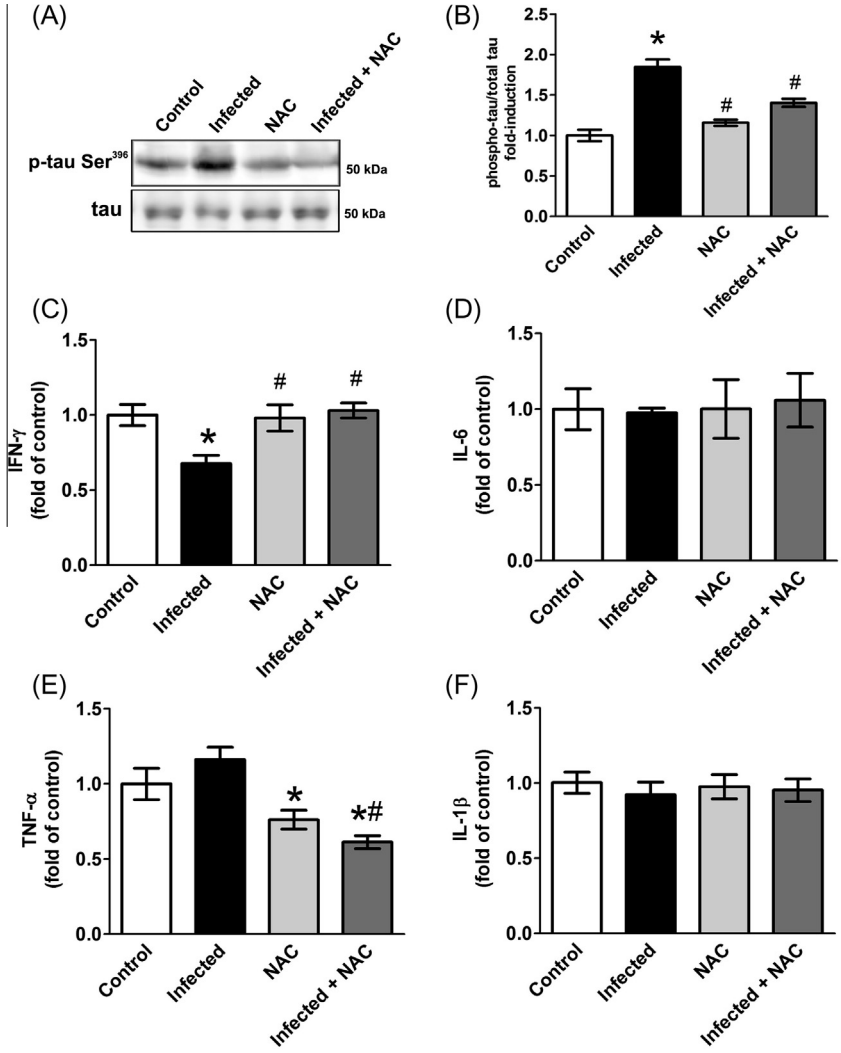

Fig. 5. Effects of antioxidant therapy with NAC in tau phosphorylation and brain cortex cytokines. Uninfected and infected animals were subdivided into groups that received one daily i.p. injection of N-acetylcysteine (NAC) 20 mg/kg for five consecutive days before the day of euthanasia. (A) Representative Western blots of brain cortex samples incubated with antibodies to tau phosphorylated at Ser396 (p-tau Ser^{396}, upper panel) and total tau (lower panel). (B) Mean ± SEM quantification values of the p-tau/total tau ratio from all samples. Relative quantification of cytokine levels was assessed by ELISA in samples from the same groups. (C) IFN-γ, (D) IL-6, (E) TNF-α and (F) IL-1β levels were analyzed. Values represent mean ± SEM of all samples from each group. ANOVA with Tukey's post hoc analysis was performed. * denotes difference from control group; # denotes difference from infected group ($p = 0.005$).

IFN-γ production. These effects are believed to be directly related to the decrease of macrophage activation and TNF-α production associated to the progression of leishmaniasis, as these events would be a consequence of the shift from Th1 to Th2 response caused by the parasite (Pereira and Alves, 2008). Thus, it is surprising that TNF-α levels are not decreased along with IFN-γ in infected animals. However, the changes in the levels of these cytokines caused by NAC treatment suggest that redox-dependent mechanisms are a key factor in the regulation of pro-inflammatory mediators at CNs level in leishmaniasis. It is possible that the pro-oxidant environment in the brain cortex of infected animals is responsible for maintaining TNF-α levels unaltered compared to control animals, instead of an expected decrease in the levels of this cytokine, since Th1 response is supposed to be suppressed.

In AD, accumulated evidence shows that stimulation of the amyloidogenic pathway results in the activation of innate immune system mainly by pattern recognition receptors (PRRs), including RAGE (Salminen et al., 2009). In the amyloidogenic pathway, beta-amyloid peptide release in CNS stimulates oligomers and fibril accumulation (early steps of amyloid plaques formation), which may act as danger-associated molecular patterns (DAMPs). DAMPs stimulate the up-regulation and activation of PRRs such as Toll-like receptors, NOD-like receptors and RAGE (Maczurek et al., 2008; Salminen et al., 2009). RAGE activation, in turn, triggers a wide array of cellular responses relevant to neurodegenerative progression in AD, such as the transcriptional activation of NF-kB-regulated genes (which include several genes associated with pro-inflammatory activation, oxidative stress and cell survival-associated responses), MAPK activation (which regulates both cell survival/death responses as well as inflammatory activation) and NADPH oxidase activation (which leads to microglia recruitment/activation, increased RS production and modulation of redox-sensitive protein kinases and transcription factors) (Arancio et al., 2004; Kojro and Postina, 2009; Maczurek et al., 2008; Sims et al., 2010; Yan et al., 2009).

Oxidative stress-related protein carbonylation, nitrotyrosination and thiol oxidation favor the formation of intra- and intermolecular protein cross-links, which leads to conformational changes increasing hydrophobicity and aggregation. These oxidative alterations favor the formation of protein cross-links, inducing generalized cellular dysfunction (Halliwell, 2006). Although we did not detect changes in thiol oxidation or nitrotyrosine formation

here, protein carbonylation was significantly increased in infected animals. This oxidative modification is responsible for the impairment in several protein functions by causing disruption of proteossomic-mediated protein turnover and generation of aberrant conformations (Kastle and Grune, 2011). We also observed that the non-enzymatic antioxidant potential in the brain cortex of infected animals was decreased, suggesting a state of enhanced RS production. These observations, altogether, strongly suggest that the increase in tau phosphorylation and RAGE protein content in the brain cortex of animals infected with *L. amazonensis* is associated with a state of oxidative stress in the CNS caused by the infection. It is possible brain oxidative stress may be a sustained response to acute pro-inflammatory activation induced in early stages of the infection, contributing later to the evolution of the chronic disease. Also, as previously stated, it is important to note that even when the presence of the parasite within the CNS is not certain, *Leishmania* spp. infection have been reported by different works to exert neurologic effects as consequence of systemic alterations originated by local inflammatory responses in organs such as liver and spleen (Melo et al., 2013; Petersen and Greenlee, 2011). In human patients with localized cutaneous leishmaniasis, plasma levels of cortisol, estradiol or prolactin positively correlated with at least one clinical parameter of the disease (lesion size, dose used to reach cure and time to cure), indicating a reflect in neuroendocrine regulation (Baccan et al., 2011). It is possible that such modifications may contribute to brain oxidative stress, since neuroendocrine hormones may exert significant changes in the redox state of the CNS (Mancini et al., 2010).

It is reasonable to suggest, based on our present data, that the increase in tau phosphorylation and RAGE immunocontent is a consequence of the oxidative stress in brain cortex. Tau phosphorylation and RAGE expression have been previously observed to be regulated by pro-inflammatory cytokines, including TNF-α and IL-1 (Li et al., 2003; Shi et al., 2011). We observed that levels of TNF-α in infected animals treated with NAC are decreased compared to infected animals that were not treated with NAC. These data indicate that a participation of TNF-α in the induction of tau aberrant phosphorylation may not be completely ruled out. On the other hand, we must consider that the decrease in IFN-γ levels could be involved in this effect, as the levels of this cytokine were recovered in infected animals subjected to antioxidant treatment. The inhibitory effect of NAC treatment on tau phosphorylation was very clear in our experimental model. These data, altogether with the effects observed other cytokines with NAC treatment, strongly suggests that aberrant tau phosphorylation is caused primarily by a redox-dependent mechanism in the brain of animals infected with *L. amazonensis*.

Several evidence associate increased RS production and oxidative damage to biomolecules to both tau aberrant phosphorylation and RAGE up-regulation/activation (de Bittencourt Pasquali et al., 2013; Mondragon-Rodriguez et al., 2014, 2013). In AD, there is a clear role of oxidative stress in the progression of neurodegeneration, and both tau phosphorylation and aggregation are involved in RS-dependent neuronal death in the course of the disease (Schmitt et al., 2012). RAGE up-regulation in response to oxidative stress was observed in different diseases where increased RS production is a characteristic, including diabetes, atherosclerosis and AD (Guo et al., 2008; Kojro and Postina, 2009). In AD, RAGE is believed to contribute in maintaining an elevated state of RS production by stimulating NADPH oxidase (Kojro and Postina, 2009), enhance tau phosphorylation via the ERK1/2-GSK3β pathway (Barroso et al., 2013; Li et al., 2012) and amyloid translocation through blood–brain barrier (Candela et al., 2010). In this context, it is possible that neurologic symptoms associated with chronic leishmaniasis are related to disruptions in the homeostasis of CNS proteins, such as tau and RAGE, as consequence of oxidative stress which in turn may be originated initially by systemic infection. This is the first demonstration of alterations in biochemical parameters of neurodegeneration in an experimental model of *Leishmania* infection. The relationship between the regulation of RAGE expression and function in the course of leishmaniasis and its relationship with modulation of Th1/Th2 responses arises from this work as an interesting issue to be addressed in future studies.

This was an exploratory study and, as such, more detailed evaluations aiming to understand mechanistic relationships or propose intervention procedures could not be performed at this stage. Therefore, it is important to point some limitations inherent to this study. First, although data presented here strongly indicate an association of oxidative stress with modulation of tau phosphorylation and RAGE in leishmaniasis, this relationship would only be fully established if administration of antioxidants is able to reverse the effects of *L. amazonensis* infection on these parameters. Also, we did not explore the full complexity of the inflammatory response, as we focused on biochemical parameters more commonly observed in inflammatory diseases of the CNS and in neurodegenerative conditions. Thus, the absence of increase in TNF-α and IL-1β levels do not necessarily mean absence of brain inflammation. A detailed study focused on a longitudinal evaluation of neurological, neurodegenerative and pro/anti-inflammatory parameters (including a wider range of Th1/Th2 mediators) must be performed for a full comprehension of the mechanisms involved in the etiology of CNS damage in leishmaniasis. Nonetheless, this is the first observation of classic neurodegenerative parameters in this disease, which is not only clinically relevant as it also opens perspectives of new mechanistic and intervention studies.

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